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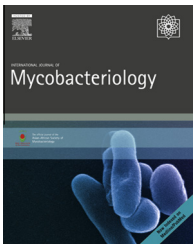

Original article

Selection of phage-displayed human antibody fragments specific for CD1b presenting the *Mycobacterium tuberculosis* glycolipid Ac_2SGL

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ABSTRACT

Objective/background: The development of new tools capable of targeting *Mycobacterium tuberculosis* (Mtb)-infected cells have potential applications in diagnosis, treatment, and prevention of tuberculosis. In Mtb-infected cells, CD1b molecules present Mtb lipids to the immune system (Mtb lipid–CD1b complexes). Because of the lack of CD1b polymorphism, specific Mtb lipid–CD1b complexes could be considered as universal Mtb infection markers. 2-Stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose (Ac_2SGL) is specific for Mtb, and is not present in other mycobacterial species. The CD1b–Ac_2SGL complexes are expressed on the surface of human cells infected with Mtb. The aim of this study was to generate ligands capable of binding these CD1b–Ac_2SGL complexes.

Methods: A synthetic human scFv phage antibody library was used to select phage-displayed antibody fragments that recognized CD1b–Ac_2SGL using CD1b-transfected THP-1 cells loaded with Ac_2SGL.

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Results: One clone, D11—a single, light-variable domain (kappa) antibody (dAbκ11)—showed high relative binding to the Ac_2SGL–CD1b complex.
Conclusion: A ligand recognizing the Ac_2SGL–CD1b complex was obtained, which is a potential candidate to be further tested for diagnostic and therapeutic applications.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), continues to be one of the leading causes of infectious disease morbidity and mortality, with reports estimating more than 1.5 million TB-related deaths annually [1,2]. Prevention of transmission remains one of the most effective control strategies, and therefore, there is an urgent need for generation of new tools useful for early and rapid diagnosis. Lipids, glycolipids, and lipopeptides derived from Mtb are presented to T cells by nonpolymorphic CD1 cell-surface molecules [3–5], thereby expanding the possible targets available to the adaptive immune system for controlling infections [5]. Patients recently infected with Mtb have CD1-restricted T-cell responses to a lipid antigen [6]. Presentation of lipid antigens to T cells has been described in TB, in association with CD1b molecules [7,8]. Importantly, a lipid antigen belonging to the group of diacylated sulfoglycolipids purified from Mtb (2-stearoyl-3-hydroxyphthiocera noyl-2′-sulfate-α-α′-D-trehalose [Ac_2SGL]) has been identified. Specific T cells recognizing the CD1b–Ac_2SGL are present in TB patients and in purified protein derivative (PPD)-positive, but not in PPD-negative, individuals [4]. The identification of cells infected with specific microorganisms is an important objective for the development of new diagnostic and therapeutic approaches. In this regard, the development of ligands with specificity to complexes of human leukocyte antigen molecules and peptide epitopes has been reported, however, the main disadvantage of this approach lies on the limited recognition spectrum associated with the high polymorphism of the major histocompatibility complex [9,10].

Because CD1b molecules are not polymorphic and Ac_2SGL is expressed only by Mtb and not by the nonvirulent mycobacterial strains, including the vaccine bacillus Calmette–Guérin, the use of CD1b–Ac_2SGL complex as a universal marker of Mtb infection should be evaluated.

Our strategy was to exploit the phage display technology to utilize its intrinsic advantages such as versatility, speed, nonuse of animals, and the possibility to recognize poorly immunogenic antigens among others [11]. A panel of antibody fragments specific for CD1b–Ac_2SGL was obtained and characterized. Such antibody fragments could be candidates for the future development of diagnostic and therapeutic tools for Mtb infection.

Materials and methods

Isolation of human antibody-displaying phages recognizing CD1b–Ac_2SGL on CD1b-transfected THP-1 cells

Phage-displayed human antibody repertoire, bacteria, and helper phage

The human single-fold scFv libraries I + J (Tomlinson I + J), *Escherichia coli* TG1, and KM13 helper phage were all kindly provided by the Medical Research Council (London, UK).

Ac_2SGL loading on CD1b-transfected THP-1 cells

Human CD1b-transfected THP-1 cells [12] were seeded in four wells of a 12-well culture plate at 10^6 cells/mL. A 10-μL volume of Ac_2SGL (20 μg/mL) was added into two wells with CD1b-transfected THP-1 cells. Cells were mixed and cultured for 4 h at 37 °C, 5% CO_2 atmosphere in Roswell Park Memorial Institute-1640 (RPMI-1640) medium without glutamine and fetal bovine serum. The unloaded CD1b-transfected THP-1 cells were used for negative selection.

Phage selection on CD1b-transfected THP-1 cells loaded with Ac_2SGL

For negative selection, unloaded cells were harvested and washed once with cold-sterile phosphate-buffered saline (PBS) and two times with 2% skim milk (M) (Sigma, USA) in cold-sterile PBS. All centrifugation steps were carried out at 4 °C at 1000*g* for 5 min in an Eppendorf plate centrifuge. The unloaded cells were incubated for 1 h at 4 °C with 10^{13} naïve phage library particles in 2 mL cold-sterile 1% M–PBS. Cells were spun down and the unbound phage-containing supernatant was used for the positive selection. The loaded cells were harvested as unloaded cells and were resuspended and incubated for 1 h at 4 °C with the preabsorbed unbound phage-containing supernatant. The cells were then centrifuged and unbound phages were eliminated by washing the wells three times with PBS.

The cells were resuspended in 500 μL of trypsin solution (Sigma, USA; 50 μg/mL) and incubated for 10 min at room temperature (RT). Cells were spun down and the bound phage-containing supernatant was carefully removed.

Selected phages were used to infect exponentially growing *E. coli* TG1 cells. Infected cells were grown overnight in 2×TY agar plates containing 100 μg/mL ampicillin and 2% glucose. Plates containing infected bacteria were scraped, and phage

particles were rescued as previously described on a 50-mL scale [13] and used as starting material for a new selection round under the same conditions as described earlier. After four selection rounds, two negative selection rounds were conducted by adding Ac_2SGL to mock THP-1 cells and unloaded CD1b-transfected THP-1 cells, respectively. Individual phage-infected colonies were picked and used to produce phagemid particles in a 96-well plate scale [12] to test their antigen recognition properties.

Phage screening

Phage-containing supernatants from 96-well plates were tested by enzyme-linked immunosorbent assay (ELISA) using cells in suspension. THP1–CD1b cells (10^5), loaded/unloaded with Ac_2SGL in 100 μL of PBS-6%–bovine serum albumin (BSA), were added to each ELISA plate well. Phage-containing supernatants (50 μL) were added and incubated (1 h at 4 °C). After two washes (PBS-4%–fetal calf serum [FCS]), horseradish peroxidase (HRP)/anti-M13 monoclonal conjugate (GE Healthcare, UK; 100 μL/1:5,000 in PBS-4%–FCS) was added and incubated (1 h at 4 °C). The plates were washed two times (PBS-4%–FCS) and 100 μL of 3,3′,5,5′-tetra methylbenzidine (TMB) substrate solution (Sigma) was added. The reaction was stopped by adding 100 μL of 1 M sulfuric acid. The absorbance (Abs) was measured at 450 nm in a microtiter plate reader (Bio-Tek Instruments). Clones with Abs ratio (loaded/unloaded cells) > 3 were considered positive.

Sequence analysis

Plasmid DNA from all positive clones was purified using QIAprep Spin Miniprep Kit (QIAGEN, USA) and the inserts were sequenced by Macrogen (Seoul, Korea) using specific primers complementary to the antibody fragment flanking sequences. Immunogenetic analysis of variable regions was performed using IMGT/V-QUEST (www.imgt.org/).

Recognition of CD1-Ac_2SGL and lipids by phage clones

Positive phage clones were rescued on a 50-mL scale and purified [13] for further evaluation.

Recognition of CD1-Ac_2SGL by phage clones

Microtitration ELISA plates (Nunc MaxiSorp, USA) were coated overnight at 4 °C with the murine anti-CD1b monoclonal antibody (mAb) BCD1b3.1 (3 μg/mL) [14] in PBS. The plates were blocked with 3% M–PBS for 1 h at RT. Recombinant human CD1b loaded with Ac_2SGL was then added at 5 μg/mL. Lipid loading onto CD1b was performed as described by Garcia-Alles et al. [15]. After 1 h of incubation at RT, three washes with PBS were carried out and 10^8 phage particles in 50 μL of 3% M–PBS were added and incubated again at RT for 1 h. After three washes, 50 μL of anti-M13 mAb–HRP (1:5,000 in 3% M–PBS) was added and incubated for 1 h. After three more washes, 50 μL of TMB substrate solution was added and incubated for 10 min at RT. The reaction was stopped by adding 1 M sulfuric acid and the Abs was measured at 450 nm. The ratio between the optical density (OD) reading against CD1b–Ac_2SGL and unloaded CD1b was determined and a threshold of positivity of 3 was established, which is above the commonly used threshold ratio of 2 [16].

Binding of phage clones to the CD1b–Ac_2SGL complex

The binding capacity of phage-selected clones was determined using microtitration ELISA plates (Nunc MaxiSorp, USA) based on two methods: (a) using serial dilutions of CD1b–Ac_2SGL as coating (5 μg/mL to 80 ng/mL) with a constant amount of phage (low-density coating ELISA) [17]; and (b) using a fixed concentration of CD1b–Ac_2SGL (coating) with serial dilutions of purified phages; the lower phage concentration required to achieve 50% of maximum binding was taken as reference for the indirect estimation of relative binding capacity [18]. Both methods were performed as described in the "Recognition of CD1-Ac_2SGL by phage clones" section.

Recognition of lipids by phage clones

Each purified phage was tested by ELISA as follows: PolySorp plates were coated with Ac_2SGL and different lipids (β-lactosylceramide, sphingomyelin, ganglioside GM1, sulfatide, disulfate sulfatide, and α-galactosylceramide; Avanti Polar Lipid, USA) in 10 μg/mL methanol and dried overnight. The plates were blocked with 3% BSA/PBS buffer for 1 h at RT. Then, 10^8 phage particles were added. After 2 h, the RT plates were washed three times with PBS. Anti-M13 mAb conjugated to HRP was added and incubated at RT for 1 h. After six washes, substrate solution addition and Abs measurement were performed as described in the "Recognition of CD1-Ac_2SGL by phage clones" section.

Production of soluble D11 (dAbκ11) and evaluation of recognition of CD1b–Ac_2SGL

Production of soluble D11 (dAbκ11)

Phagemid DNA from D11 phage clone was purified using QIAprep Spin Miniprep Kit (QIAGEN) and was transformed in *E. coli* BL21 (DE3). A single colony was grown at 37 °C in 50 mL of 2×TY medium containing 2% glucose and 100 μg/mL ampicillin until OD_{600} of cultures reached 0.6. Cells were then harvested and grown for 6 h at 28 °C in 50 mL of 2×TY with 100 μg/mL ampicillin and 1 mmol/L isopropyl-D-thiogalactopyranoside. After centrifugation, periplasmic extract was obtained using the osmotic shock method. In brief, the cell pellet was resuspended in 1 mL of ice-cold TES buffer (0.2 mol/L Tris–HCl, 0.5 mmol/L EDTA, 0.5 mol/L sucrose, pH 8.0) and 15 mL of cold 0.2×TES was subsequently added. After incubation on ice for 30 min, the suspension was centrifuged and periplasmic fraction containing the supernatant was obtained and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Periplasmic fraction from a 100-mL culture, previously equilibrated in binding buffer (BB; 50 mM NaH_2PO_4 and 300 mM NaCl), was used as the starting material for purification by immobilized metal affinity chromatography with Ni-Chelating Sepharose Fast Flow (GE, USA). The resin was washed with BB containing 20 mmol/L imidazole. The antibody fragments were eluted with BB containing 250 mmol/L

imidazole. The specific fraction containing soluble dAbκ11 was desalted against PBS on a PD-10 column (GE, USA) and analyzed by SDS-PAGE. Protein concentration of the final sample was determined by BCATM Protein Assay Kit (Pierce, USA) according to manufacturer's instructions.

Recognition of CD1b–Ac_2SGL by dAbκ11

Serial dilutions (5 μg/mL to 130 ng/mL) of purified soluble dAbκ11 in PBS were added to a microtiter MaxiSorp ELISA plate previously coated with the CD1b–Ac_2SGL complex and unloaded CD1b as described in the "Recognition of CD1-Ac_2SGL by phage clones" section. After 1-h incubation at RT, the plate was washed with PBS-T and 9E10 mAb (directed to the c-myc tag) (Sigma Aldrich, USA) at 10 μg/mL in PBS-3%–BSA was added for 1 h at RT. After washing, an antimouse immunoglobulin G–HRP (Sigma) diluted 1:3000 in PBS-3%–BSA was added for 1 h at RT. After washing, substrate solution was added and Abs measurement was carried out as described in the "Recognition of CD1-Ac_2SGL by phage clones" section.

Results

Isolation of phages recognizing CD1b–Ac_2SGL on CD1b-transfected THP-1 cells

Antibody-displaying phages were selected using Ac_2SGL-loaded CD1b-transfected THP-1 cells. Clones producing phage-displayed antibody fragments able to recognize CD1b–Ac_2SGL on CD1b–THP-1 cells were identified by cell suspension ELISA screening. To exclude nonspecific binding, the phage clones were also tested against unloaded CD1b–THP-1 cells. While most of the analyzed phage clones bound non-specifically to unloaded CD1b–THP-1 cells or showed a ratio of loaded/unloaded cells > 3 (data not shown), eight clones preferentially bound Ac_2SGL-loaded CD1b–THP-1 cells (Fig. 1). They were obtained after four rounds of panning and two additional rounds of negative selection.

The eight phage clones recognizing CD1b–THP-1 cells were sequenced. After sequence analysis, all clones except D11 corresponded to the expected scFV structure. D11 was determined to be a single, light-variable domain (kappa) antibody (dAbκ11).

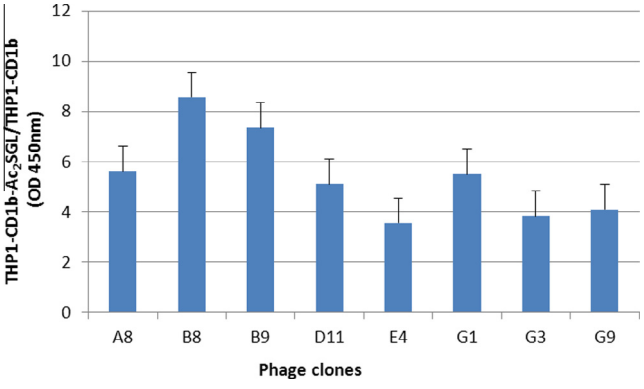

Fig. 1 – Human antibody-displaying phages recognizing CD1b–Ac_2SGL on CD1b-transfected THP-1 cells. Supernatants containing phage clones were tested by cell suspension ELISA using Ac_2SGL-loaded and Ac_2SGL-unloaded CD1b-transfected THP-1 cells. Bound phages were detected by HRP–anti-M13 mAb. The ratio of the developed color on loaded and unloaded CD1b-transfected THP-1 cells was plotted for each phage clone. Optical density ratio > 3 was considered positive. pHEN1 phagemid clone-containing supernatant was used as the negative control. Bars indicate standard deviation. Ac_2SGL = stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody.

Recognition of CD1b–Ac_2SGL and different lipids by phage clones

Recognition of CD1b–Ac_2SGL by phage clones

The recognition of the selected phage clones was tested using soluble CD1b–Ac_2SGL complexes coated onto ELISA plates. All the phage clones recognized the CD1b–lipid complex over unloaded CD1b (Fig. 2). D11 (dAbκ11) showed the highest reactivity to loaded CD1b.

Binding of phage clones to CD1b–Ac_2SGL

Binding and relative affinity of phage clones were evaluated using either serial dilution of coated CD1b–Ac_2SGL or serial dilutions of phage clones with a specific concentration of coated CD1b–Ac_2SGL. All the phage clones demonstrated binding to CD1b–Ac_2SGL in the experimental conditions assayed, and D11 (dAbκ11), B8, and B9 showed the best binding capacities (Fig. 3A and B).

Recognition of different lipids by phage clones

The recognition of uncomplexed Ac_2SGL and other glycosphingolipids (β-lactosylceramide, sphingomyelin, ganglioside

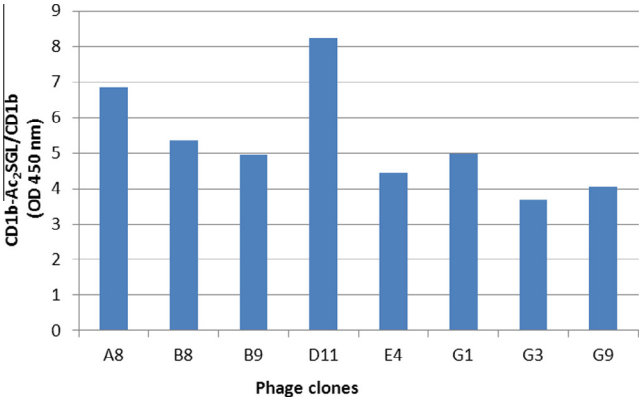

Fig. 2 – Recognition of the CD1b–Ac_2SGL complex by the phage-displayed antibody fragments. ELISA plates were coated with Ac_2SGL-loaded and Ac_2SGL-unloaded soluble CD1b. Bound phages were detected with HRP–anti-M13 mAb. Absorbance ratios (loaded/unloaded cells) > 3 were considered. Ac_2SGL = Stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody.

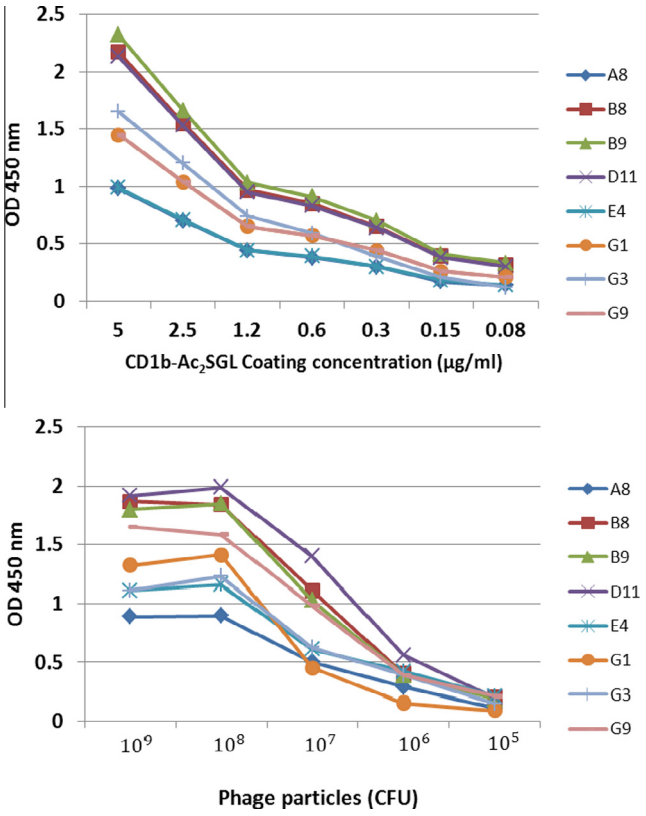

Fig. 3 – Evaluation of CD1b–Ac_2SGL binding of phage clones and relative binding capacity. Relative binding capacity was evaluated using (A) serial dilutions of the CD1b–Ac_2SGL complex (coating) and a fixed amount of phage particles (10^8), or (B) different concentrations of phage clones and constant concentration of the coating CD1b–Ac_2SGL complex. In both assays, bound phages were detected with HRP–anti-M13 mAb. Ac_2SGL = stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose; CFU = colony forming units; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody.

GM1, sulfatide, disulfate sulfatide, and α-galactosylceramide) by phage clones was studied by ELISA. All phage clones showed specific recognition of Ac_2SGL (Fig. 4).

Recognition of CD1b–Ac_2SGL by soluble D11 (dAbκ11)

The D11 (dAbκ11) clone was selected to be further evaluated in its soluble form taking into consideration the specific recognition of CD1b–Ac_2SGL, the binding relative affinity, and its single-domain structure, which is advantageous for future potential applications.

Single-domain antibody dAbκ11, derived from the D11 clone, was expressed as a soluble antibody fragment in the periplasmic extract of BL21 (DE3) cells and purified. The reactivity of dAbκ11 with CD1b–Ac_2SGL versus unloaded CD1b was confirmed using purified antibodies (Fig. 5).

Discussion

The development of molecules specific for Mtb-infected cells has important implications because these tools may facilitate understanding of the mechanisms regulating host–pathogen interactions *in vivo*. In addition, the development of new tools capable of discriminating Mtb-infected cells may have potential applications in the diagnosis, treatment, and prevention of TB. An ideal tool should detect markers that are only produced by Mtb and are independent of the genetic background and polymorphisms of infected cells. Because of the lack of CD1b polymorphism, Mtb lipid–CD1b complexes can be considered as universal Mtb infection markers [5]. In this regard, sulfoglycolipids are specifically found in Mtb and among them, Ac_2SGL is antigenic and presented by CD1b [4].

A few mAbs against Mtb lipids obtained by hybridoma technology are now available [19–21]. The production of a ligand obtained from a human Fab antibody library recognizing the human CD1d–α-galactosylceramide (α-GalCer) complex [22] has been reported. These Fab fragments specifically detected the soluble complexes as well as those on antigen-presenting cells in culture and *in vivo* in dendritic cells from transgenic mice [22].

In this study, we used a human synthetic naive library to isolate antibody fragments against CD1b–Ac_2SGL complexes. Phage-displayed antibody fragments were selected using CD1b-transfected THP-1 cells loaded with the Mtb sulfoglycolipid Ac_2SGL. We decided to use this system to display the glycolipid antigen on the surface of human antigen-presenting cells trying to reproduce, as much as possible, the conditions in which the CD1b–Ac_2SGL complex is presented *in vivo*. A panel of eight phage-displayed antibodies was obtained using our approach. All of them were able to specifically recognize the CD1b–Ac_2SGL complex over unloaded CD1b. At the same time, all the selected phage clones recognize Ac_2SGL not complexed to CD1b, with no recognition of other lipid molecules, which adds extra potential versatility to these ligands. Previously, we obtained similar results with a filamentous phage displaying a *T-cell receptor* (TCR) fragment specific for this complex [23]. Regarding the recognition of plastic-bound Ac_2SGL, it should be noted that such recognition was less intense than the recognition of the CD1b complexes. This finding could be related to the recognition of lipid regions also available for interaction with the antibody when the lipid is included in the complex.

One interesting result was the unexpected selection of a single-variable light-domain antibody using a synthetic scFv library. It represents a "defective" clone that only received the light-variable chain during the construction of the library.

Single-domain antibodies represent an interesting alternative for diagnosis and therapy due to their potential advantages such as stability, tissue penetration, low immunogenicity, and recognition of cryptic epitopes among others [24–27]. In the case of single-domain variable light-chain antibodies, although less studied, several reports highlight their potential advantages and usefulness in diagnosis and therapy [28–32].

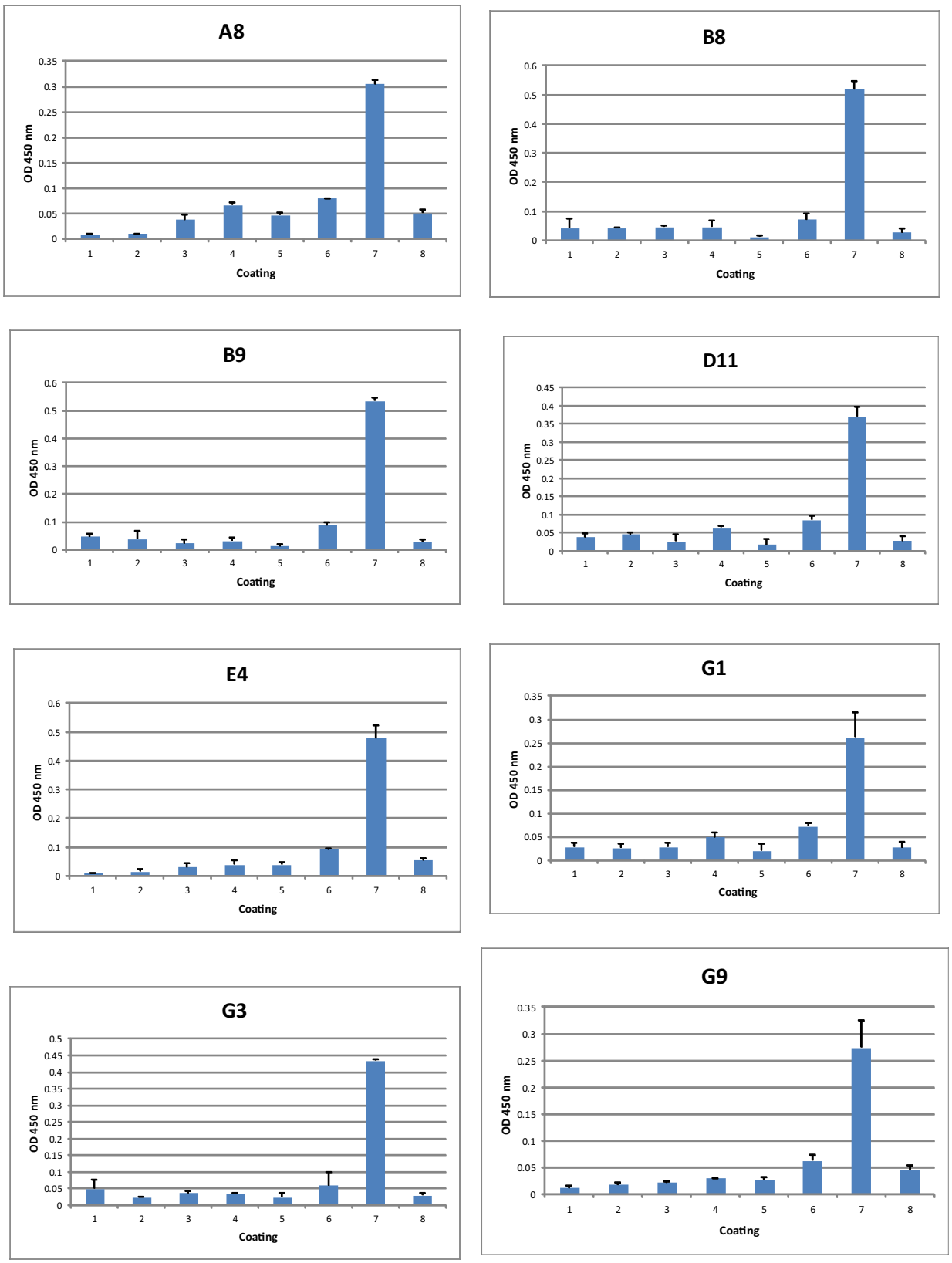

Fig. 4 – Recognition of different lipids by phage clones (A8, B8, B9, D11, E4, G1, G3, and G9) using lipid-coated plates. Bound phages were detected with HRP–anti-M13 mAb. 1 = β-lactosylceramide, 2 = sphingomyelin, 3 = ganglioside GM1; 4 = sulfatide; 5 = disulfate sulfatide; 6 = α-galactosylceramide; 7 = Ac_2SGL; 8 = phosphate-buffered saline. Bars indicate standard deviation. Ac_2SGL = stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody.

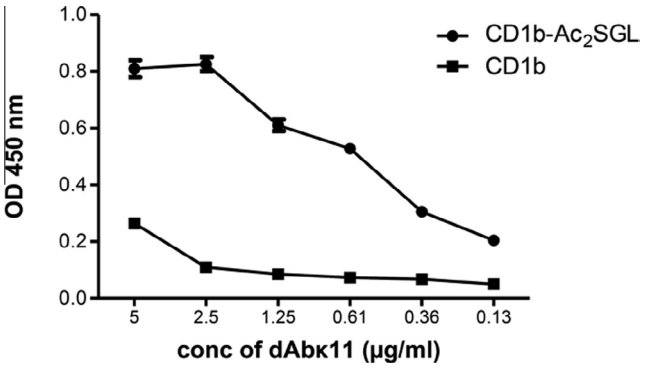

Fig. 5 – Soluble, single-domain antibody dAbκ11 recognizes the CD1b–Ac_2SGL complexes. Immunoreactivity of purified dAbκ11 against recombinant CD1b–Ac_2SGL complexes. Bound antibody fragments were detected by ELISA using HRP-labeled 9E10 mAb. Results are presented as OD_{450} nm ± standard deviation. Ac_2SGL = stearoyl-3-hydroxyphthioceranoyl-2′-sulfate-α-α′-D-trehalose; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody; OD = optical density.

Considering the potential importance of the immune response to mycobacterial lipid antigens presented by CD1b molecules [5,7,33,34], and the identification of specific CD1b–Mtb lipid complexes associated with Mtb infection [4], the use of TCR-like structures recognizing these complexes for the development of diagnostic and therapeutic tools for TB is an option that deserves to be explored in the future.

The high relative binding capacity and specificity of the D11 (dAbκ11) phage clone for the CD1b–Ac_2SGL complex and its recognition of free Ac_2SGL support using this clone for further development of diagnostic and therapeutic applications.

In conclusion, we have identified human antibody fragments capable of recognizing the CD1b–Ac_2SGL complex. These antibodies might be evaluated as potential diagnostic, therapeutic, and research tools. The simple phage-selection method we have developed can be expanded to obtain larger panels of human antibody fragments in the search for functional antibodies against different CD1b–mycobacterial lipid complexes.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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